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Mesenchymal Stromal/Stem Cell– or Chondrocyte-Seeded Microcarriers as Building Blocks for Cartilage Tissue Engineering

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In this study we have tested the use of mesenchymal stromal/stem cell (MSC)– or chondrocyte (hch)–laden microcarriers as building blocks for engineered cartilage tissue. MSCs and hchs expanded on microcarriers were used in chondrogenic coculture and compared with monoculture of MSCs or hchs. The use of cell-laden microcarriers as building blocks for cartilage tissue engineering led to a compact tissue formation with significant volume increase compared to the biomaterial-free approach. After 28 days of differentiation culture, formation of cartilaginous matrix in cocultures and chondrocyte monoculture approaches was observed. Coculture resulted in beneficial glycosaminoglycan deposition compared with monoculture of MSCs or chondrocytes attached to microcarriers. Further, the microcarrier-adhered coculture displayed increased levels of the differentiation marker ACAN and reduced levels of the dedifferentiation marker COL1A1. To our knowledge, this is the first article that successfully combines an innovative combination of cell expansion on microcarriers and the direct use of MSC- or hch-cell-laden microcarriers as building blocks in cartilage tissue engineering.

Introduction

In 1743, William Hunter described for the first time the avascular structure of cartilage, as well as its limited capacity for self-repair.[1] Until today, cartilage repair and regeneration after damage is only possible to a limited extent. Tissue engineering (TE) therefore offers possibilities for optimization of cartilage repair by combining different cell types, biomaterials, and growth factors for the support of cartilage regeneration. For cellular approaches, two of the best-described cell sources for cartilage repair are chondrocytes and mesenchymal stromal/stem cells (MSCs). Human chondrocytes (hchs) for autologous chondrocyte implantation (ACI) are mainly obtained from biopsies of the nonweight bearing parts of the joint. After harvesting, they need to be expanded to achieve sufficient cell numbers to fill and regenerate critical size defects. Traditionally, monolayer chondrocyte cell expansion is associated with the loss of phenotype along with a decreased capacity to secrete extracellular matrix over increasing passage numbers. Therefore, scientists look into alternative expansion and culture strategies to overcome the described limitations.

In 1996, Frondoza *et al.* introduced a new culture method for chondrocytes—the expansion on commercially available microcarriers. This improved the yield, as well as the conservation of chondrogenic phenotype. Until now, microcarriers have been successfully used in several approaches to expand, redifferentiate, and differentiate chondrocytes.[2–5] It was shown that gelatin- or dextran-based microcarriers with highly porous structures especially support the round chondrocyte phenotype. Nevertheless, microcarriers are as well used to culture other cell sources, including liver cells,[6,7] embryonic stem cells,[8] and, most relevant for cartilage tissue engineering, MSCs.[9] Notably, expansion of MSCs on microcarriers improved proliferation and differentiation and can be used to guide and improve differentiation.[9–11]

Performance of the cell types in cartilage tissue engineering can be as well augmented by exploiting coculture strategies. Cocultures of different cell sources are based on the idea that multisignal events *in vivo* cannot be perfectly mimicked by adding a limited variety of growth factors to a monoculture. In this way, cells are exposed to a wider variety of stimuli, mimicking *in vivo* conditions. In cartilage tissue engineering, MSCs were initially used to reduce the amount of chondrocytes needed or to omit their use all together. Interestingly, *chondro-induction* was observed in cocultures of chondrocytes and MSCs; superior neocartilage

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was formed by the combination of the two different cell types as compared with either cell type alone.[12,13] This phenomenon can only to a limited extend be explained by the induction of chondrogenic differentiation of MSCs by articular chondrocytes.[14,15] It was demonstrated that MSCs act as trophic mediators and enhance chondrocyte proliferation and differentiation.[12,15–17] This coculture strategy reveals the possibility of omitting *in vitro* expansion of chondrocytes in traditional ACI procedures. Combining freshly harvested chondrocytes with MSCs may lead to a single-step surgery for cartilage treatment, in which chondrocytes are isolated during the surgery, mixed with bone marrow cells or expanded MSCs from the same patient, loaded on a scaffold, and directly re-implanted into the patient. The additional immunomodulatory role of MSCs might even allow for allogenic coculture repair strategies.[18]

Nevertheless, the applicability of using only cells for filling middle-sized to large cartilage defects is limited. For improved clinical practice it is important to transfer single-cell models to advanced tissue engineering strategies by including assisting biomaterials. Main purpose of the scaffold is the provision of structural support by optimal filling the entire defect and allowing attachment, proliferation, and differentiation of cells in a three-dimensional (3D) environment. The mechanical support provided by the scaffold can lead to a decrease in the rehabilitation time for the patient. Nowadays, tissue-engineered scaffolds are as well employed to mimic the natural environment by employing natural materials (hyaluronan,[19] collagens,[20] and fibronectin[21]) to create a favorable microstructure for cellular homing. The porous gelatin microcarriers used in this article provide supporting structures for cellular attachment and homing[22] and have been described as optimal expansion strategy for MSCs and chondrocytes.[23]

Combining innovative coculture approaches and the favorable characteristics of cellular expansion of cells on microcarriers, we demonstrate for the first time, how small units of chondrocyte- or MSC-laden gelatin microcarriers can directly serve as building blocks for tissue culture constructs. Further we demonstrate, how these building blocks serve as an easy and efficient for the transfer of coculture approaches from single-cell level to a complete tissue engineering strategy.

Materials and Methods

Cell culture and expansion

The use of human bone marrow aspirates and human knee biopsies was approved by a local Medical Ethics Committee. Human primary chondrocytes (hchs) were obtained from full-thickness cartilage dissected from total knee biopsies (femoral condyle and tibial plateau) of a patient undergoing knee replacement as published previously.[24] In short, the harvested cartilage was digested overnight in 0.15% collagenase type II solution. After digestion, hchs were washed and cultured up to passage one on tissue culture plastic in chondrocyte proliferation medium (Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 0.2 mM ascorbic acid 2-phosphate, 0.4 mM proline, 100 U penicillin/mL, and 100 mg/mL streptomycin). Hchs were characterized for their ability to deposit chondrogenic matrix as well as their mRNA expression of COL2A1. MSCs from the bone marrow of three female donors undergoing hip replacements (average age = 64 years) were isolated from aspirates as described previously.[25] MSCs were selected by adherence in proliferation media [α-MEM, 10% fetal bovine serum (Lonza), 0.2 mM ascorbic acid, 2 mM L-glutamine, 100 U/mL of penicillin, 100 mg/mL streptomycin, and 1 ng/mL of basic fibroblast growth factor (Instruchemie)]. MSC surface markers were tested CD73, CD90, and CD105 positive and negative for HLA-DR, CD45, CD34, CD11b, and CD19.

After one passage of two-dimensional (2D) expansion, MSCs or hchs were seeded on Cultisphere G microcarriers (Percell) and were cultured for 10 days in proliferation medium in stirrer flasks (500 mL). In short, for 1×10^6 cells, 0.1 g of Cultisphere-G was rehydrated in PBS and autoclaved and then transferred to 100 mL of cell suspension in proliferation media. For the first 48 h the cells were cultured at 25 rpm for 30 s and 30 min pause. After 48 h, 100 mL of proliferation media was added and stirring was changed to continuous mode at 50 rpm. For the reference culture without microcarriers, MSCs were cultured in monolayer at a density of 2500 cells/cm^2.

All data are averages of three separate experiments using three different MSC donors in combination with the same hch donor.

Microcarrier construct culture

At day 10 of culture, aliquots of the chondrocytes and MSCs attached to microcarriers were trypsinized and counted to determine the cell number per milliliter of microcarrier solution. Culture conditions with 1×10^6 cells per construct were prepared according to Table 1. Tissue culture constructs were formed by seeding microcarrier-free single-cell suspensions and cell-laden microcarrier suspensions in hanging well inserts (Millipore; Ø 6 mm) as described previously.[26] Single-cell suspensions were derived after trypsinization of cells

Table 1. Culture Scheme for the Comparison of Microcarrier-Cell Combinations and Single-Cell-Seeded Constructs

Condition	*MSCs*	*Chondrocytes*
100% MSC mc	Attached to microcarrier	None
80% MSC mc/20% hchs	Attached to microcarrier	3D-expanded single-cell solution
100% hch mc	None	Attached to microcarrier
100% MSC 2D	3D-expanded single-cell solution	None
80% MSC 2D/20% hchs	3D-expanded single-cell solution	3D-expanded single-cell solution
100% hchs	None	3D-expanded single-cell solution

MSCs, mesenchymal stromal/stem cells; 3D, three-dimensional; 2D, two-dimensional.

expanded on microcarriers. In an additional condition the MSC-seeded microcarriers were mixed with single-cell suspensions of chondrocytes in a cell–cell ratio of 4:1 to translate the culture model into a coculture strategy. Identical cell numbers were used in each culture condition. Our strategy is depicted in Figure 2A.

Cultures were maintained in chondrogenic differentiation media (Dulbecco's modified Eagle's medium supplemented with 40 mg/mL of proline, 50 mg/mL ITS-premix, 50 mg/mL of ascorbic acid, 100 mg/mL of sodium pyruvate, 100 U penicillin/mL and 100 mg/mL streptomycin, 10 ng/mL of transforming growth factor-beta, and 10^{-7} M of dexamethasone) for 28 days. Earlier time points of the presented constructs were not feasible, since limited tissue connectivity did not allow for proper handling of the construct before day 21.

Live–dead staining

Single microcarriers after 10 days of expansion culture or for microcarrier constructs after 28 days of culture were washed in PBS and stained for 30 min at 37°C in 6 μM ethidium homodimer/1 μM calcein. Images of 3D microcarrier constructs were taken by confocal microscopy (LEICA LSM510) whereas images of the single microcarrier constructs were taken with a Nikon (E600) upright fluorescence microscope.

Scanning electron microscopy

After 28 days in culture, samples were fixed in 10% formalin, dehydrated in increasing series of ethanol, critical point dried, mounted on a sample holder, and gold sputtered. Samples were broken in half after snap freezing in liquid N_2 during the dehydration procedure. Imaging acquisition was done with Philips XL 30 ESEM-FEG scanning electron microscope (10 V).

Rheology

After 28 days in culture, rheological experiments were carried out with an MCR 301 rheometer (Anton Paar) using parallel-plate (25-mm diameter) configuration at 37°C in the oscillatory mode. The plate distance was set to 2 mm and samples were measured at increasing frequency (1–10 Hz) and increasing strain (1–10%). During measurements the measuring chamber was sealed and filled with oil to avoid drying of the sample.

Histology

After 28 days in culture, cell constructs were fixed with 10% buffered formalin for 15 min and embedded in paraffin using routine procedures. Sections of 5 μm were cut and stained for sulfated glycosaminoglycans (GAGs) with Alcian blue (0.5%, in H_2O, pH = 1 adjusted with HCl, 30 min) combined with counterstaining of nuclear fast red (0.1% in 5% aluminum sulfate, 5 min).

Wet weight and dry weight; quantitative GAG and DNA assays

After 28 days in culture, constructs were washed with phosphate-buffered saline and shortly dipped on filter paper to remove excess liquid before weighing. Samples were freeze dried overnight and weighed again to determine their dry weight. Subsequently samples were digested and GAG content was determined as described previously.[27] Cell number was determined via quantification of total DNA with CyQuant DNA kit according to the manufacturer's description and fluorescent plate reader (Perkin Elmer).

RNA isolation and quantitative polymerase chain reaction

Total RNA was isolated from pellet culture with the Nucleospin RNA II kit (Bioke) after 28 days in culture. Up to one microgram of total RNA was reverse-transcribed into cDNA using the iScript cDNA Synthesis kit (Bio-Rad). The primers for quantitative polymerase chain reaction (qPCR) were designed with primer blast and are listed in Table 2. B2M and RLP13 were used as house-keeping genes for normalization as described earlier for chondrocyte-containing cultures.[28] We used following protocol for amplification: denaturation at 95°C for 10 min; 44 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 15 s; and melt curve: 55°C

Table 2. Human Primers Used for Quantitative Polymerase Chain Reaction Analysis

Gene symbol	*Primer sequence*	*Amplicon length*	*T(a)*
ACAN	5′ AGGCAGCGTGATCCTTACC 3′ 5′ GGCCTCTCCAGTCTCATTCTC 3′	136 bp	64°C
COL1A1	5′ GTCACCCACCGACCAAGAAACC 3′ 5′ AAGTCCAGGCTGTCCAGGGATG 3′	121 bp	60°C
COL2A1	5′ CGTCCAGATGACCTTCCTACG 3′ 5′ TGAGCAGGGCCTTCTTGAG 3′	122 bp	60°C
COL10A1	5′ GCAACTAAGGGCCTCAATGG 3′ 5′ CTCAGGCATGACTGCTTGAC 3′	129 bp	56°C
SOX9	5′ TGGGCAAGCTCTGGAGACTTC 3′ 5′ ATCCGGGTGGTCCTTCTTGTG 3′	98 bp	60°C
RPL13	5′ AAAAAGCGGATGGTGGTTC 3′ 5′ CTTCCGGTAGTGGATCTTGG 3′	101 bp	60°C
B2M	5′ GACTTGTCTTTCAGCAAGGA 3′ 5′ ACAAAGTCACATGGTTCACA 3′	106 bp	60°C

to 95°C with 0.5°C increment for 5 s per step. QPCR results were analyzed using the $\Delta\Delta^{-ct}$ method.

All reagents were purchased from Invitrogen unless otherwise stated. Common chemicals were purchased from Sigma-Aldrich.

Statistical analysis

Statistical analysis was performed using a one-way ANOVA followed by a *post-hoc* Tukey test. Significances of $p \leq 0.05$ are indicated (*).

Results

Cells expanded on microcarriers can be used as building units for cartilage TE constructs

Here we prove that chondrocytes retained their round morphology when expanded on microcarriers in stirrer flasks after 10 days of culture (Fig. 1A, B). During expansion on microcarriers, both cell types maintained full viability as shown by calcein/ethidium homodimer staining (Fig. 1C, D). We performed kinetics of the cell culture on microcarriers previously and compared them to 2D tissue culture plastic culture. Cellular growth was comparable in both culture methods within the 10 days of culture (data not shown).

We next explored whether chondrocyte- or MSC-laden microcarriers or combinations there off could be directly used in tissue engineering of cartilage tissue. For this, cell-laden microcarrier constructs were cultured in hanging well inserts. Cartilage tissue formation was compared with single-cell-seeded wells (Fig. 2A).

After 28 days of culture, small discs of microtissues with an average height of < 1 mm were produced in hanging wells seeded with single-cell suspensions (Fig. 2B, bottom). In contrast, stable microtissues with an average height of 4 mm were produced in hanging well inserts seeded with MSC-laden microcarriers only or with MSC-laden microcarriers in combination with a single-cell suspension of chondrocytes in a cell–cell ratio of 4:1 (Fig. 2A, bottom). Macroscopically there was no obvious morphological difference between the latter two conditions. We next examined the effect of using cell-laden microcarriers to build 3D constructs for cartilage tissue engineering on cell viability. After 28 days of differentiation the 3D constructs were stained for living and dead cells and cross-sectioned, and confocal pictures of the bottom, middle, and top part were taken. Viable cells were present in each compartment of the tissue construct, with variation between the different zones (Fig. 3A, B). Cell numbers in different areas of the construct were evenly distributed in constructs containing chondrocytes whereas constructs consisting of MSC-laden microcarriers contained significantly lower amount of cells in the middle part of the construct (Fig. 3C). In each of the three seeding regimes, considerable cell death, in particular in the middle of the construct, was noted after 28 days of culture (Fig. 3B). Cell death was most pronounced in constructs built of MSC-laden microcarriers. Mixing the MSC-laden microcarriers with a single-cell suspension of chondrocytes slightly improved cell viability particularly in the middle compartment of the construct. Highest cell survival was observed in constructs build of chondrocyte-laden microcarriers. Remarkably, while cell death was most pronounced in the middle compartment of constructs containing MSC-laden microcarriers, most dead cells were found in the top and bottom compartments of the constructs build of chondrocyte-laden microcarriers (Fig. 3B).

Cartilage, like many soft connective tissues, is exposed to a wide spectrum of loading and shear stress. The high water content makes cartilage a viscoelastic tissue. The elasticity is displayed via fluid–solid frictional dissipation and pressurization of the fluids in the tissue. We therefore measured the viscoelastic behavior of the hydrogel-like microcarrier constructs by determining the storage and loss modulus that represent values for the elastic portion and the viscous portion of a construct. Rheological analysis revealed minor differences between the different constructs with a storage modulus of about 750 Pa and an about 10-fold lower loss modulus when 10 Hz of stress or 10% strain was applied

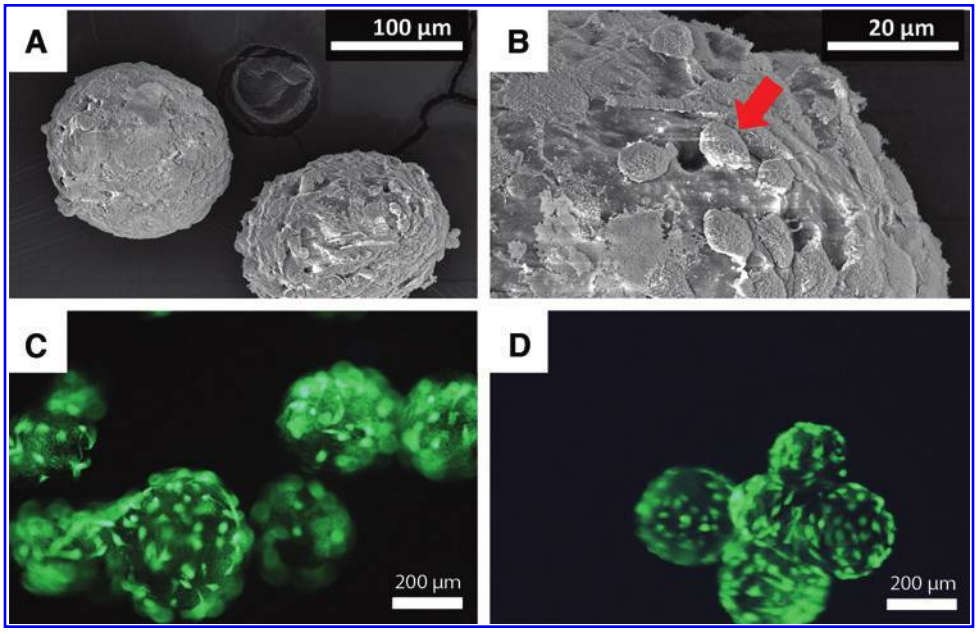

FIG. 1. Expansion of chondrocytes and mesenchymal stromal/stem cells (MSCs) on microcarriers. **(A)** Scanning electron microscopy picture of a microcarrier laden with hchs. **(B)** Higher magnification of **(A)** showing that hchs maintained their round phenotype (red arrow), **(C)** MSCs and **(D)** hchs showing high viability after expansion on microcarriers as demonstrated by a live–dead staining, in which living cells fluoresce green and dead cells fluoresce red after 28 days of expansion culture. Color images available online at www.liebertpub.com/tea

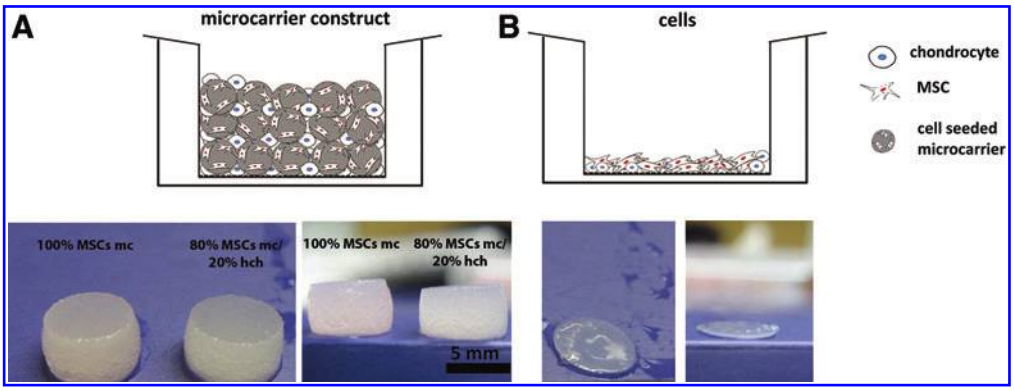

FIG. 2. Efficient macrotissue formation using cell-laden microcarriers as building blocks. **(A)** Cell-laden microcarriers were cultured in hanging inserts alone or were mixed with single-cell suspension of three-dimensional-expanded hchs and cultured in hanging inserts for 28 days in differentiation medium as shown in the top. Four-millimeter-thick tissue constructs were obtained in each of the two conditions as shown at the bottom. **(B)** Three-dimensional-expanded single-cell MSCs were seeded in hanging inserts and cultured in differentiation medium as shown in the top. After 28 days of culture thin discs of tissue were obtained as shown in the bottom. Color images available online at www.liebertpub.com/tea

(Fig. 4A, B, respectively). Similar results have been obtained for gelatin gels before by Kalyanam *et al.*[29]

Microcarrier-based tissue constructs form cartilaginous tissue

All conditions of tissue-engineered constructs using cell-laden microcarriers as building blocks were then subjected to scanning electron microscopy (SEM). SEM analysis displayed compact tissue formation at the lower part of the constructs and more loose tissue in the upper part (Fig. 5A, error indicating bottom of the construct). All microcarrier constructs revealed similar results. At higher magnifications the round and compact structure of the microcarriers could still be recognized easily (Fig. 5B). The cell-laden microcarriers were surrounded and connected by extracellular matrix [Fig. 5B, C (arrow)]. The microcarriers provided cellular niches for (in) growth and differentiation of the cells in the construct (Fig. 5D).

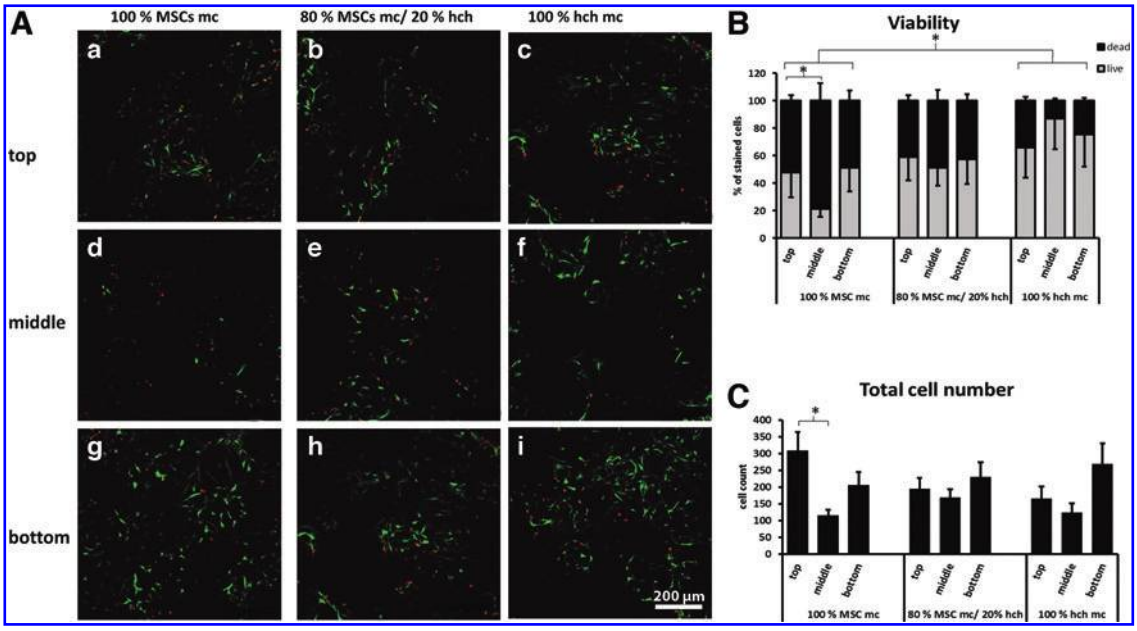

FIG. 3. Cell viability in the microcarrier tissue constructs. **(A)** Tissue constructs of cell-laden microcarriers [100% MSCs mc (left), 80% MSCs mc mixed with 20% 3D-expanded single hch (middle), or 100% hch mc (right)] were obtained after 28 days of culture in differentiation medium in hanging inserts. A live (FDA, green) and dead (PI, red) staining of cross-sections was performed. The top row is a representative picture out of three repeats of the top zone of the construct **(a–c)**. The middle row is representative for the middle section of the construct **(d–f)** and the bottom row is representative for living and dead cells in the bottom zone of the tissue construct **(g–i)**. **(B)** Quantification of living and dead cells in the top, middle, and bottom zones of the tissue constructs in **(A)**. Data represent the mean ± SD of three independent experiments. **(C)** Quantification of total cell numbers present in each zone of the tissue constructs. Data represent the mean ± SD of at least three independent experiments. $*p < 0.05$. Color images available online at www.liebertpub.com/tea

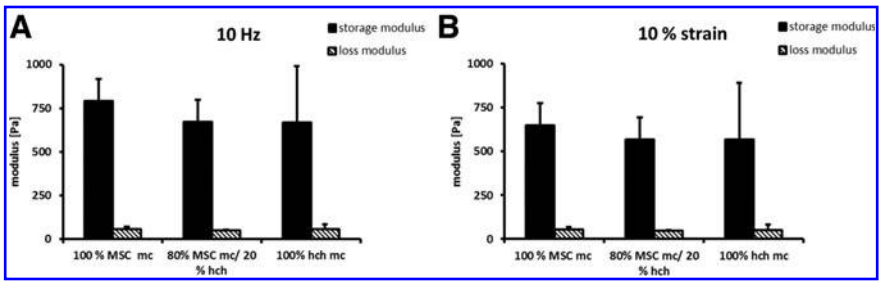

FIG. 4. Rheological analysis of the microcarrier-based tissue constructs. Storage and loss modulus of cell-laden microcarrier-based tissues (various cell combinations) were determined at a stress of 10 Hz **(A)** or a strain of 10% **(B)**. Data represent the mean of three independent experiments ± SD.

Staining for GAG deposition demonstrated cartilaginous matrix formation. In the single-cell-seeded constructs, a thin layer of packed cells surrounded by Alcian-blue-positive GAGs was observed (Fig. 6A, F, L). In all three conditions cartilage formation was observed. The chondrocyte-based constructs had a rather fibrous cartilage morphology with stretched lacunae (Fig. 6L). As expected, staining appeared more intense in the coculture of MSCs and hchs in line with previous observations.[12]

In contrast to the single-cell-seeded constructs, the microcarrier constructs displayed heterogeneous extracellular matrix deposition. Constructs solely consisting of MSC-laden microcarriers hardly stained positive for Alcian blue, while intense staining was found at the bottom of the construct of MSC-laden microcarriers mixed with chondrocytes or chondrocyte-laden microcarriers [Fig. 6B, G, M (arrows); C, H, N). This staining gradually decreased when moving from bottom to top with a slight increase in staining at the top layer (Fig. 6E, K, P). At higher magnifications, matrix formation inside the hollow structures of the microcarriers was observed, indicating first signs of microcarrier degradation as well as cellular ingrowth in the microcarriers (Fig. 6C, H, N). The amount of matrix formation was lower in the MSC-laden microcarrier culture, whereas staining in cocultures of MSC-laden microcarriers and hchs tended to be higher than in chondrocyte monoculture. The cell density in the microcarrier-based constructs was lower than in the according single-cell constructs, resembling a more cartilage-like phenotype.

For validation of histological data, biochemical quantitative analysis was performed. In agreement with their bigger size, the wet weight of microcarrier-seeded constructs was about 20-fold higher compared with single-cell-seeded constructs. Also the dry weight of the microcarrier-containing constructs was higher (Fig. 7A, B). In both cases the increased weight was the result of the contributing mass of the microcarriers to the construct. Quantitative DMMB-based GAG measurements of three different

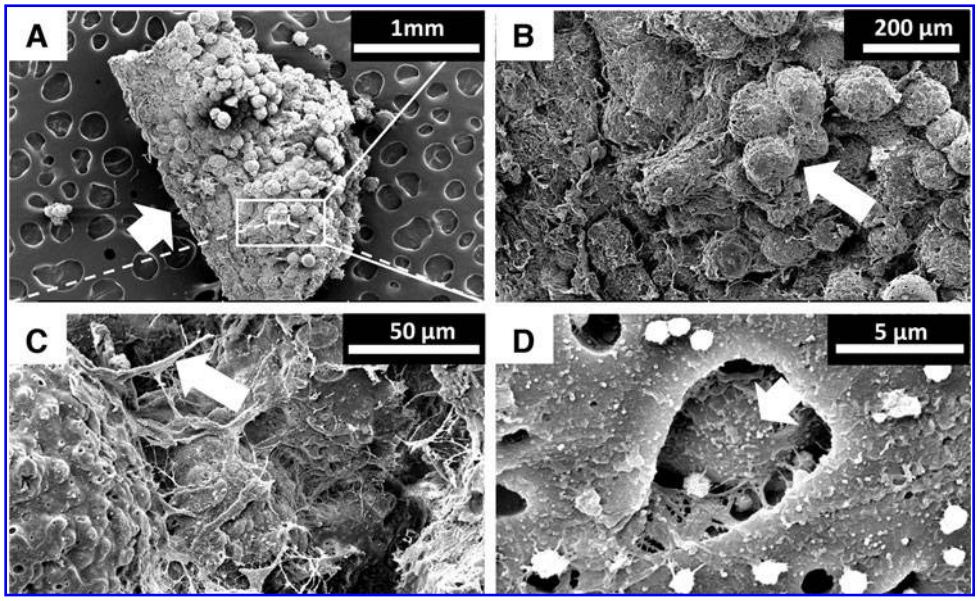

FIG. 5. Scanning electron microscopy (SEM) of the microcarrier tissue constructs after 4 weeks of culture. **(A)** Scanning electron microscopy showed compact tissue formation at the lower part of the construct (arrow) and more loose tissue with visible single microcarriers in the upper part. **(B)** Higher magnification of the boxed region in A showing individual microcarriers interconnected by cells and matrix. The arrow points to four microcarriers visibly connected by protein fibers. **(C)** Further magnification of **(B)** showing the round outline of microcarriers (arrow) that are visibly connected by matrix **(D)** whereas it is difficult to distinguish individual cells on microcarriers. SEM clearly provided evidence for the presence of cellular niches at the surface of the microcarrier that might be favorable for growth and differentiation. The arrow points at a cell homing in a microcarrier, whereas the light dots around the cells likely represent protein aggregates secreted by cells homing to the microcarriers. Representative picture out of three independent experiments is shown.

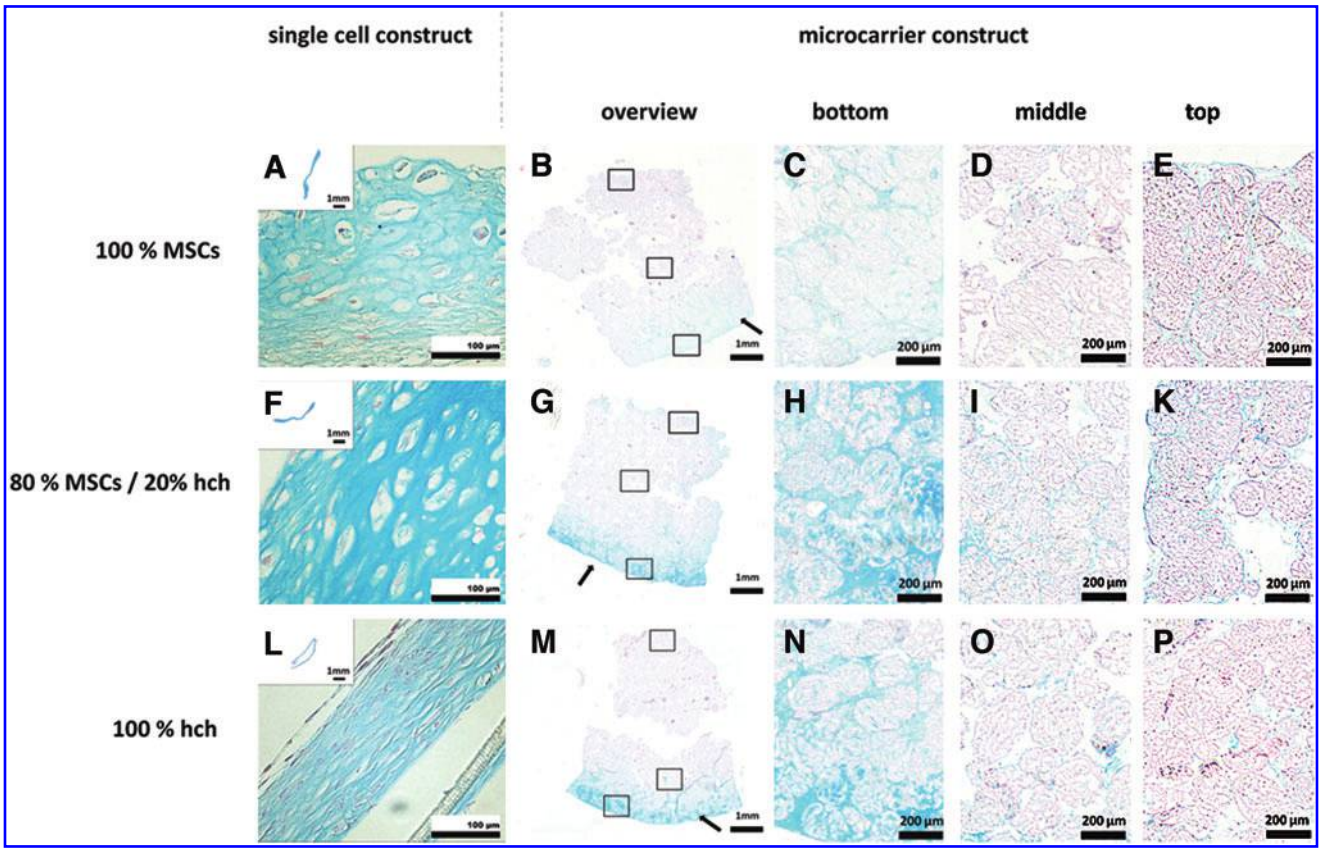

FIG. 6. Alcian blue stain of glycosaminoglycan (GAG) deposition in the microcarrier construct compared with the cellular constructs. **(A, F, L)** Histological sections show that single-cell-seeded constructs result in thin layers of tissue with Alcian blue stain in all conditions. High magnifications show more fibrous cartilage formation in the hch tissue **(L)** whereas MSCs and coculture led to round chondron-like cellular morphology inside the matrix **(A, F)**. **(B–E, G–I, K, M–P)** Microcarrier constructs show cartilaginous matrix formation at the bottom of the construct and lowest abundance of staining in the middle of the constructs. **(H, N)** Matrix formation inside the microcarriers is visible in the coculture of 80% MSCs and 20% hch as well as the 100% hch culture. **(F, G, H, I, K)** The cocultured constructs show a more intense Alcian blue stain in single-cell cultures as well as cell-laden microcarrier cultures. Representative pictures out of three independent experiments are shown. Color images available online at www.liebertpub.com/tea

donors confirmed the lowest cartilage formation in MSC microcarrier cultures, and revealed a trend to increased GAG formation in the coculture approach although this did not reach significance. Single-cell-seeded constructs displayed the highest GAG deposition in the MSC cultures whereas cocultures and chondrocyte cultures showed comparable cartilaginous tissue formation after correction for DNA (Fig. 7C). DNA content of cultures could give an indication about proliferation or cell death in the cell culture approach. Although seeding same total cell numbers in the beginning, the DNA content of single-cell-seeded constructs was always lower than of microcarrier-seeded constructs. This suggested that the microcarrier environment better supported cell proliferation and/or cell survival than single-cell-seeded constructs. The DNA levels were more stable in microcarrier-based constructs than in single-cell-based constructs.

Reduced markers of dedifferentiation and hypertrophy in microcarrier-seeded constructs

Subsequently we analyzed gene expression levels in single-cell- and microcarrier-seeded constructs by qPCR. Comparison of single-cell constructs and microcarrier constructs showed higher mRNA expression of the marker *SOX9* (significant) and *COL2A1* in single-cell-seeded constructs (Fig. 8A, D). *ACAN* was expressed to the same amount in coculture of both culture approaches (Fig. 8E). *COL1A1*, a marker of dedifferentiation, was expressed significantly higher in single-cell conditions (Fig. 8B), compared with microcarrier-based constructs. The ratio between *COL2A1* and *COL1A1* was not different (Fig. 8B). *COL10A1* mRNA was nonsignificantly reduced in MSC-laden microcarrier-based constructs compared with single-cell-based constructs. No *COL10A1* mRNA expression was detected in constructs based on hchs (Fig. 8F).

Discussion

Previous publications have demonstrated that MSCs and chondrocytes can be expanded on microcarriers with the same or even improved efficiency.[5,10,30] With the here-described microcarrier-assisted culture set-up, we enable the transfer of MSC-and-chondrocyte coculture to a full cartilage tissue engineering strategy. We present a method that offers the possibility to be applied in coculture-based single-step cartilage repair strategies in clinical practice.

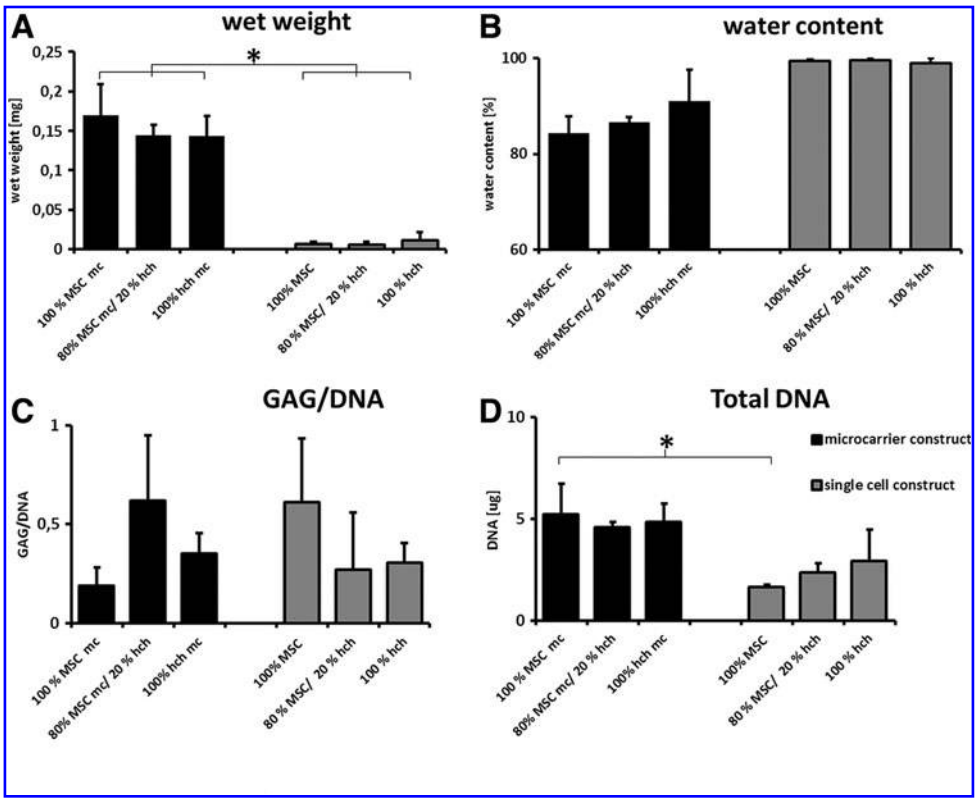

FIG. 7. Biochemical analysis of the microcarrier-based culture in comparison with the cellular construct. **(A)** Wet weight of the microcarrier tissues is significantly higher than in the cellular tissues resembling the bigger volume of the tissues. **(B)** Microcarrier tissues contain less water than cellular constructs when normalized with construct weight. **(C)** The coculture approach results in higher relative glycosaminoglycan deposition in the microcarrier culture, whereas in the cellular approach MSCs seem to deposit more GAGs (not significant). **(D)** Total DNA content is in general higher in the microcarrier-containing constructs with significant difference in the MSC-containing samples. Data represent the mean ± SD of at least three independent experiments. $*p < 0.05$.

The coculture approach has been described to enable beneficial cartilage formation, namely, *chondro-induction*, marked by increased GAG deposition; decreased cell dedifferentiation, as measured by *COL1A1* gene expression; and decreased hypertrophic cell differentiation leading to less calcification/endochondral bone formation *in vivo*.[12,31,32] The beneficial effect of coculture was assigned to soluble factors released by MSCs. Recently, we identified the MSC-released FGF-1 as key protein inducing the enhanced chondrogenesis in cocultures of MSCs and hchs.[17] For further improvement

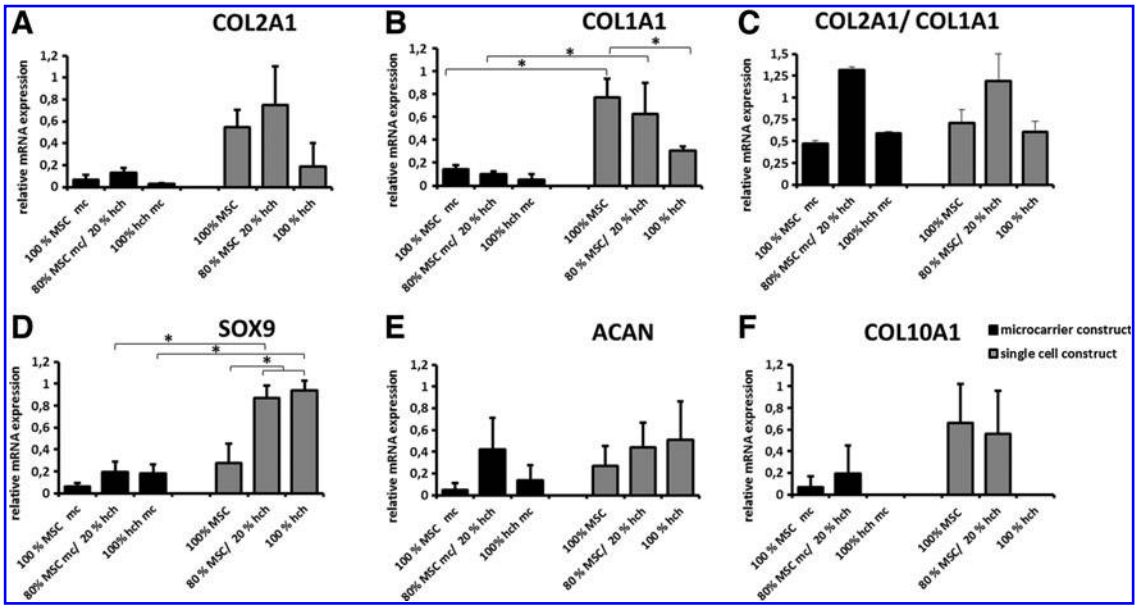

FIG. 8. Relative mRNA expression in microcarrier- and single-cell-based constructs. **(A, D, E)** Chondrogenesis markers *COL2A1* and *SOX9* are higher in cellular constructs in all experimental conditions; whereas *ACAN* is expressed to the same amount in the coculture approach of both culture approaches. **(B, C)** Despite the fact that COL1A1 is significantly higher expressed in single-cell cultures, no difference between microcarrier and cellular constructs was observed when the ratio of COL2A1/COL1A1 (ratio of differentiation vs. dedifferentiation is formed). **(F)** COL10A1 is only expressed in the coculture and the MSC samples. Data represent the mean ± SD of at least three independent experiments. $*p < 0.05$.

of cartilage repair strategies, it is important to bring this coculture strategy from the bench to the bedside.

Coculture might be a suitable strategy to overcome the low availability of chondrocytes for repair. For the improvement of ACI, it is favorable to omit the step of chondrocyte expansion and assist the limited amount of freshly isolated primary chondrocytes with a second cell type. The here-described expansion of MSCs on hollow microcarriers has been proven to improve MSC proliferation[10] and provides microenvironments for homing of MSCs (Fig. 5D). For microcarrier-assisted cocultures, autologous MSCs can be taken from iliac crest, expanded on microcarriers, and used in the surgery with freshly isolated chondrocytes.

We further demonstrate that microcarriers seeded with MSCs or chondrocytes can directly serve as building blocks for tissue-engineered cartilage constructs. In contrast to constructs seeded with equal numbers of single cells, relatively thick, up to 4 mm, cartilaginous tissue constructs can be obtained. Currently, research groups either characterize novel microcarriers for cartilage tissue engineering[33,34] or use the combination of previously 2D-expanded cells and microcarriers.[35,36] To the best of our knowledge, we are the first to combine microcarrier cell expansion and their direct use as building blocks for cartilage tissue engineering. Our approach might be applicable for cartilage defects but also for other trauma's in which relatively large defects need to be filled. A similar approach has been used before in bone tissue engineering. Instead of gelatin-based microspheres, cell-laden calcium phosphate particles were used to fill up critical bone size defects.[37] Thus, by selecting the appropriate material for microsphere generation, best fitting with characteristics of the desired tissue, this approach could be amenable for the tissue engineering of a wide variety of tissues.[34,38]

Coculture of MSCs attached to microcarriers and 3D-expanded chondrocytes led to improved GAG deposition, as well as a decrease in *COL1A1* expression. We were able to confirm the nonsignificant downregulation of *COL10A1* in this culture model, whereas a decreased level of calcification would have to be tested in an *in vivo* model. The combination of the superior performance of cocultures as well as the microcarrier-based size increase of the construct makes this concept an optimal strategy to overcome the limitations of single-cell-based cartilage tissue engineering. The microcarrier-based culture model displayed lower expression values for *COL2A1* and *SOX9* when compared with single-cell constructs. Remarkably, the overall DNA levels revealed a higher cell number in microcarrier-containing constructs, underlining that lower per cell *SOX9* and *COL2A1* levels might be compensated by more cells expressing respective genes.

The tissue obtained by cell-laden microcarriers has a hydrogel-like appearance (Fig. 2A), which results mainly from the ability of the gelatin microcarrier to swell in water. The use of microcarriers further increases the volume of the engineered constructs and supports thereby the filling of bigger defects. Healthy cartilage consists of 75–85% of water and thereby it is desirable to engineer cartilage with biomaterials that offer similar physical composition.[39] The here newly engineered tissue consists of 80–90% of water and has thereby a desirable feature of engineered cartilage (Fig. 7B). These characteristics are as well underlined by the rheological measurements that are in the range of gelatin hydrogels described in the literature.[29]

An often-observed problem in the use of collagen-based hydrogels is contraction of the whole construct depending on the forces the cells develop as well as the degradation process of the biomaterial. Gelatin microcarriers are known to have a highly crosslinked structure that degrades very slowly. In histological Alcian blue staining, we observed cartilaginous matrix formation inside the microcarriers, which appeared more hollow and might have undergone first degradation by enzymes secreted by the ingrowing cells (Fig. 6H, N). Even though degradation was seen in histological stainings, the microcarriers mainly exhibited their round morphology, in particular, in the top part of the construct, as was seen by scanning electron micrographs (Fig. 5A). This indicated that the highly crosslinked structure of the gelatin-based Cultisphere-G microcarriers likely enables long lasting support and will thereby allow further stabilization of the defect meanwhile facilitating tissue formation. In different tissue engineering approaches using the same type of microcarriers, degradation took about 8 weeks.[3] For the proof of principle, these microcarriers provided sufficient support in the cartilage tissue engineering construct. Nevertheless, an optimization of degradation time might lead to even faster matrix deposition and improved chondrogenesis. The production of gelatin microcarriers with different degrees of crosslinking has been shown to have an influence on cell proliferation, survival, and differentiation of mouse fibroblasts.[40] Evidence suggests that microspheres with longer degradation times have a positive effect on these parameters. Reasonably lower degrees of gelatin crosslinking will allow faster degradation of the gelatin scaffold and thereby create more space for chondrogenic matrix deposition. Nevertheless, it is important to allow a certain time frame for degradation. In case, degradation is faster than matrix deposition, the constructs might be too fragile and lose their integrity in the defect site during loading.[41]

In the here-described microcarrier constructs, viability was lower in middle of the constructs when MSCs were used as major cell source. Constructs consisting of single chondrocyte had a higher viability in all areas of the construct. In 3D cultures, microcarriers can be used as a porogen for better nutrient supply, which enables the formation of thicker tissue constructs. This key feature of microcarriers enables cells to survive in all parts of the constructs, whereas chondrocytes seem to have an optimized range of adaptation for reduced nutrient supply. Malda *et al.* described that nutrient deprivation can activate chondrogenesis.[42] This might indicate that chondrocytes with their limited nutrient supply in mature cartilage are more adopted to survival in 3D constructs than MSCs with limited nutrient supply and waste product exchange in the center of the constructs. By additional comparison of DNA content of the microcarrier-assisted culture with single-cell culture construct, it was clear that cell numbers in microcarrier constructs after 28 days of differentiation culture were higher. Higher cell numbers were likely the result from better proliferation conditions, offered by the presence of the microcarriers.[10,30] The increased surface area minimizes competition between cells for limited cell attachment places.

We here underline that cell-laden microcarriers can be used in cartilage coculture strategies. Especially for ACI strategies, cell-laden microcarriers can assist to overcome the limitations of chondrocyte availability, homogeneous cell distribution in the defect, and efficient filling of the lesion. Additionally, during ACI, the coculture set-up can improve the quality and quantity of chondrogenic matrix formation.

Nevertheless, for optimal cell differentiation, nutrient supply, and degradation regimes, it is wise to optimize the used microcarriers according to the engineered tissue requirements. Solorio *et al.* demonstrated that the continuous release of transforming growth factor-beta from microspheres can improve chondrogenic differentiation of MSCs and promote their survival.[36] This approach might overcome nutrient limitations in the center of the construct and further improve the presented approach. Further, the use of less-crosslinked gelatin or poly(lactic-co-glycolic acid) as biomaterial can reduce degradation times and might be better for chondrogenic matrix deposition.

Conclusion

In conclusion in this article we demonstrate that cell-laden microcarriers used for the expansion of either MSCs or hchs can be directly used as building blocks for coculture approaches in tissue-engineered constructs. They may have potential for direct application in biomaterial-assisted cartilage repair strategies without the need of trypsinization and isolation of microcarrier-expanded single cells.

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Disclosure Statement

No competing financial interests exist.

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